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PRELIMINARY AMENDMENT
NS Entry of PCT/EP2003/012839

AMENDMENTS TO THE SPECIFICATION

Please amend the specification at page 1, after the title and before line 4, by inserting:

This Application is a 371 of PCT/EP2003/012839, filed November 17, 2003; the disclosure of which is incorporated herein by reference.

Page 3, lines 4-18, are amend as follows:

Prideaux ~~(~~(The 16th International Pig Veterinary Society Congress, Melbourne (Australia) 17-20th September 2000, pag.439-442)) describes a vaccine prepared from a strain with an inactivated *apx*IIC gene that secretes and express a non-activated *Apx*II toxin unable therefore to attach to the target cells.

So, the live attenuated vaccines described in the previous background of the invention, based on App strains without haemolytic capability, are less immunoprotective because they have suffered modifications in their structure that do not allow them to attach to the membrane receptor of the target cells. Furthermore these can not generate antibodies against *Apx*I and/or *Apx*II toxins, since these are not secreted by the cell. Frey et al. (Gene ~~(~~142:97-102 (1994)) describe the amino-acid sequence of the ApxI exotoxine from a serotype I strain and Smiths et al.;~~((~~Infection and Immunity 59:4497-4504 (1991)) describe the amino-acid sequence of the ApxII exotoxin of a serotype 9 strain.

W 12-8-09 **Page 3, lines 22-~~27~~ 26, are amended as follows:**

The authors of the present invention have discovered a method to obtain an immunogenic and non-haemolytic App strain from an App virulent strain which has been modified in at least one segment of *apx*IA gene (SEQ ID NO 1) and optionally in a segment of the *apx*IIA gene (SEQ ID NO 2) which code a

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transmembrane domain of the Apx cytolytic and haemolytic exotoxins.

ILV/12.8.05 **Page 4, lines ~~24-33~~ 23 32, are amended as follows:**

Fig. 1 shows an alignment performed with the ClustalX program (Thompson et al; Nucleic acid Research **24**:4876-4882 (1997)) between the amino-acid sequences of ApxI coming from a serotype 1 strain (Frey et al; Gene **142**: 97-102 (1994)) and ApxII from serotype 9 (Smits et al.; Infection & Immun. **59**:4497-4504 (1991)). In this figure only the sequence contained between amino-acids 1 to 594 of ApxI and 1 to 590 of ApxII have been enclosed. On the alignment the following regions are framed: H1 (amino-acids 233 to 253), H2 (amino-acids 296 to 315) and H3 (amino-acids 369 to 405). These regions correspond respectively to the three transmembrane domains present in both Apx.

Page 5, line 26 to Page 6, line 8, are amended as follows:

Figure 3 is divided in three panels: Panel A shows the restriction maps in kilobases (kb) and the distribution of the genes in the operon *apx*I from the genome of App. In light gray, the *apx*IA gene is depicted, being the target of the different recombination events, and in dark gray the adjacent genes *apx*IC, *apx*IB and *apx*ID. The different genes or regions of plasmid pApxIΔH2 are drawn using skewed bars. The coding fragments of the transmembrane helices (H1, H2 and H3) of apxIA are highlighted in Black. The names and some detailed structures in figure 2 plasmids have been simplified. Thus gfpUV comprises the ptac promoter and the atpE/GFPUV fusion; OriV indicates the vegetative origin of replication of R6K and OriT the origin of transference by conjugation of RP4. In (1) and (3) both are shown the restriction map obtained with

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enzyme *Xho*I and the distribution of the genes of operon *Apx*I of the App genome are shown. In (2) the restriction map of the same operon after the insertion of plasmid pApxIΔH2 in the App genome is shown. This insertion occurs by a unique homologous recombination event between flanking regions 5´ of H2 placed in plasmid pApxIΔH2 and the App genome respectively.

lW 12.8.09 **Page 10, lines ~~5-17~~ 4 16, are amended as follows:**

The transmembrane domains, present in the *apx*IA and in *apx*IIA genes of the haemolytic and cytolytic exotoxins, were detected using the Transmem and Helixmem programmes above mentioned. The prediction performed on the amino-acid sequences of the haemolytic and cytolytic exotoxins ApxI and ApxII indicate that the transmembrane domains, also named transmembranes, are found located in the following zones of the sequence of the exotoxins:

- First transmembrane domain H1: between amino-acids 233 and 253 corresponding to the nucleotides 697~~699~~ to 759 from *apx*I.
- Second transmembrane domain H2: between amino-acids 296 and 315, corresponding to nucleotides 886~~888~~ to 945 from a*px*I
- Third transmembrane domain H3: between amino-acids 369 to 405, corresponding to nucleotides 1105~~1107~~ to 1215 from *apx*I

lW 12.8.09 **Page 10, lines ~~21-29~~ 20 28, are amended as follows:**

The modification is carried out, preferably, by deletion of the nucleotides 886~~885~~ to 945~~944~~ of the *apx*IA gene which code the second transmembrane domain of the App *Apx*I exotoxin.

Another preferred realization, of the method object of the present invention, furthermore introduces an additional deletion in the segment of *apx*IIA gene which codes the second

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transmembrane domain of the ApxII exotoxin of App. Preferably a deletion of nucleotides 886~~885~~ to 945~~944~~ of gene *apx*IIA, which code the second transmembrane domain of the App *Apx*II exotoxin.

WP 12-5-09 **Page 12, lines ~~18~~ 17-~~27~~ 25, are amended as follows:**

In the present invention the obtained mutant AppApxIH2$^-$ is identical to the original wild type App strain, except for the deletion of nucleotides 886~~885~~ to 945~~944~~ (both inclusive) of the coding sequence of gene *apx*IA which corresponds with that absence of the amino-acids 296 to 315 (both inclusive) in the produced ApxI.

In the present invention the obtained mutant AppApxI/IIH2$^-$ is identical to strain AppApxIH2$^-$ except by the deletion of nucleotides 886~~885~~ to 945~~944~~ (both inclusive) of the coding sequence of gene *apx*IIA which corresponds with the absence of amino-acids 296 to 315 (both inclusive) in the ApxII produced.

Page 13, lines 18-30, are amended as follows:

Another object of the invention is an App strain characterized because it has a deletion in nucleotides 886~~885~~ to 945~~944~~ of the *apx*IA gene, that code the second transmembrane of the ApxI exotoxin, deposited in the Colección Española de Cultivos Tipo (Spanish Collection of Type Cultures) with the registration number CECT 5985, according to the Treaty of Budapest of 28th April 1977, or a mutant thereof.

Another object of the invention is an App strain characterized by having a deletion of nucleotides 886~~885~~ to 945~~944~~ of the *apx*IA gene that code the second transmembrane of the ApxI exotoxin and besides a deletion of nucleotides 886~~885~~ to 945~~944~~ of *apx*IIA gene that code the second transmembrane of the ApxII exotoxin deposited in the Spanish Collection of Type

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Cultures with the registration number CECT 5994, according to the Budapest Treaty, or a mutant thereof.

Page 15, lines 4-16, are amended as follows:

The techniques and DNA recombinant methods applied as follows, are described in detail in Sambrook and Russell (In Molecular cloning 3rd Ed. Cold Spring Harbor Laboratory Press, Cold spring Harbor New York (2001) and Ausubel et al; *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc. (1998)). All PCR products were previously cloned in a pBE plasmid before being digested with restriction enzymes. This plasmid is a derivative of pBluescript SK2 (Stratagene) vector and presents the multiple cloning site substituted by a small nucleotidic sequence which specifies only the target of the restriction enzyme *Eco*RV.

The *E. coli* XL1-blue strain (Stratagene) has been used as a host for hybrid vectors based on plasmids pUC118 or pBluescript SK. The *E. coli* S17-1 λ pir strain (~~[~~(Simon et al; Biotechnology **1**:784-791 (1983)) has been used as a host of the hybrid vectors based in plasmid pGP704.

W/12809 **Page 15, line ~~32~~ 30 to Page 16, line ~~10~~ 9, are amended as follows:**

The three transmembrane domain which adopt an α- helix structure, were determined by means of the use of programmes TransMem ~~[~~(Aloy *et al*; Comp. Appl. Biosc. **13**:213-234 (1997)) and Helixmem ~~[~~(Eisenbeg *et al*; J. Mol. Biol. 179: 125-142 (1984)) as described for *E. coli* ~~[~~(Ludwig *et al*; Mol. Gene. Genet. **226**:198-208 (1991)) applied to the amino-acid sequence of the ApxI coming from a serotype 1 strain ~~[~~(Frey *et al*; Gene **142**: 97-102 (1994)) and the ApxII of a type 9 serotype ~~[~~(Smits *et al*; Infection and Immunity **59**:4497-4504 (1991)). These programmes detected three regions which could act as

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transmembrane helices in both proteins (Fig 1): the first transmembrane is located between amino-acids 233 and 253 (H1); The second transmembrane is located between amino-acids 296 and 315 (H2) and the third transmembrane between amino-acids 369 and 405 (H3) all of them from ApxI.

Page 16, lines 20-26, are amended as follows:

Plasmid pGP704 ~~(~~(Miller and Mekalanos; J. Bact. **170**:2575-2583 (1988)) was cut simultaneously with restriction enzymes *Bgl*II and *Eco*RI. Using electrophoresis in agarose gel a 3.7 kb DNA fragment was isolated. This fragment incubated in a ligation reaction together with oligonucleotides pGP5′ (GAT CGA ATT CAG GAT ATC ACA GAT CT) (SEQ ID NO ~~1~~3) and pGP3′ (ATT TAG ATC TGT GAT ATC GTG AAT TC) (SEQ ID NO ~~2~~4). The obtained recombinant plasmid was named pGP1.

WP 12.8.09 **Page 16, line ~~32~~ 31 to Page 17, line ~~17~~ 16, are amended as follows:**

Using plasmid pMAL-p2 (New England Biolabs) the sequences corresponding to promoter ptac were amplified by PCR using the ptac5′oligonucleotide primers (GAA TTC AAT GCT TCT GGC GTC AG) (SEQ ID NO 5~~3~~) and ptac3′ (GGT ACC GGA TGA GAT AAG ATT TTC) (SEQ ID NO 6~~4~~) which enclose respectively the restriction targets *Eco*RI and *Kpn*I in its 5′ends. Also from pMAL-p2 plasmid, by PCR the sequences corresponding to the rho-independent terminator of operon *rrn*B were amplified using the primer oligonucleotides *rrn*B5′ (GGT ACC GGA TGA GAT AAG ATT TTC) (SEQ ID NO 7~~5~~) and *rrn*B3′ (GAA TTC AAG AGT TTG TAG AAA CGC) (SEQ ID NO 8~~6~~) which enclose respectively the restriction targets *Kpn*I and *Eco*RI in their 5′ ends. The size of the DNA amplified fragment comprises 278 base pairs (bp).

With the plasmid pAG408 ~~(~~(Suarez *et al*; Gene **196:** 69-74 (1997)) a fusion of the gene of the GFPUV protein with the